UNIVERSITI PUTRA MALAYSIA

IN VITRO FERTILIZATION OF HAMSTER OVA AS AN AID IN ASSESSING THE FERTILIZING CAPACITY OF HUMAN SPERMATOZOA

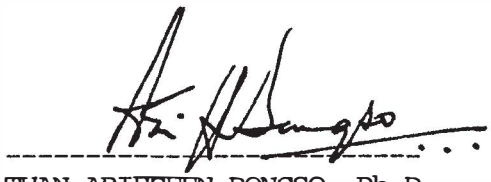
SYED ZULKIFLI BIN SYED MOHAMED

It is hereby certified that we have read this thesis entitled "In Vitro Fertilization of Hamster Ova as an Aid in Assessing the Fertilizing Capacity of Human Spermatozoa" by Syed Zulkifli bin Syed Mohamed, and in our opinion it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirements for the degree of Master of Science.

ALANG P. ZAINUDDIN, Ph.D.
Assoc. Professor/Dean of Graduate Studies
Universiti Pertanian Malaysia
(Chairman Board of Examiners)

GORDON JAMES KING, Ph.D.
Professor
Department of Animal Science
University of Guelph
Canada
(External Examiner)

ADNAN SULONG, Ph.D.
Lecturer
Faculty of Veterinary Medicine and Animal Science
Universiti Pertanian Malaysia
(Internal Examinar)



TUAN ARIFFEEN BONGSO, Ph.D.
Associate Professor
Faculty of Veterinary Medicine and Animal Science
Universiti Pertanian Malaysia
(Internal Examiner/Supervisor)

This thesis was submitted to the Senate of Universiti Pertanian Malaysia and was accepted as partial fulfilment of the requirements for the degree of Master of Science

Date: 19 JUN 1986

ALANG P. ZAINUDDIN, Ph.D.
Associate Professor/
Dean of Graduate Studies

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by

SYED ZULKIFLI BIN SYED MOHAMED

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science in the Faculty of Veterinary Medicine and Animal Science Universiti Pertanian Malaysia

This thesis is dedicated to my parents

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IN VITRO FERTILIZATION OF HAMSTER OVA AS AN AID IN ASSESSING THE FERTILIZING CAPACITY OF HUMAN SPERMATOZOA

by

Syed Zulkifli bin Syed Mohamed

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Supervisor : Associate Professor Dr. Tuan Ariffeen Bongso

Faculty : Veterinary Medicine and Animal Science

Studies have been recently undertaken in many research laboratories to examine the fertilizing capacity of spermatozoa from human males with histories of unexplained infertility to investigate their ability to penetrate ova. Since human ova are not readily available, hamster ova have been used as a substitute for these studies and research is going on to test the validity of this assay as a reliable diagnostic tool for examining sperm-ovum interaction in man and animals.

A study was undertaken to develop such an assay system for evaluating the causes of 'unexplained infertility' in males at the National Population and Family Development Board in Kuala Lumpur.

The semen characteristics of 175 ejaculates from 35 donors (one ejaculate per donor per week) showed 10 fertile parients to posses normal semen parameters and 25 infertile patients to have azoospermia (4%), oligospermia (44%), poor sperm counts (32%), normozoospermia (12%) and polyzoospermia (8%). Ten of the infertile patients with semen parameters within normal limits were put to the sperm penetration assay. Thirty mature cycling female golden hamster (Mesocricetus auratus) were superovulated using 30 IU pregnant mare's serum gonadotropin on the day following oestrus followed 48 hours later with 30 IU human chorionic gonadotrophin (HCG), both drugs being administered intraperitoneally. Uterine and oviductal flushings taken 15-17 hours after the injection of HCG gave 30±1.03 ova (20-43) for each hamster. Ham's F10 medium was found to be suitable for maintenance of ova and in vitro fertilization. To obtain zona-free ova, the cumulus cells and zona pellucida were removed with 0.1% hyaluronidase for 5-6 mins at 37°C and 0.1% trypsin for 2-3 mins at 37°C respectively. Eighty percent of zona-free ova were fertilized with sperm (1.5×10^6 sperm/ml) from fertile donors, when the semen was washed with Ham's F10 medium and preincubated at 37°C for 6 hours ($P<0.05$). Penetration rates were significantly lower ($P<0.01$) for zona-intact and cumulus-intact ova at sperm preincubation times of 3 to 9 hours. When sperm preincubation was fixed at 6 hours, maximum penetration rates of 80.0±2.9% were observed when zona-free ova were allowed to interact with sperm for 6 hours ($P<0.05$). Penetration rates in zona-intact and cumulus-intact ova were significantly lower ($P<0.01$) than zona-free ova when sperm-ovum interaction was 6 hours. Penetration

of zona-free ova was not observed in 60% of 'infertile' patients with normal semen characteristics, even when ejaculates per week per patient were used over a 5 week period. Chromosome analysis was possible in 95% of penetrated ova and the presence of discrete haploid sets of human sperm chromosomes in the hamster ooplasm was used to confirm penetration. The results of this study demonstrate that the zona pellucida of the hamster ovum acts as a barrier to human sperm and zona-free hamster ova may be a useful substitute for human ova to test the fertilizing capabilities of spermatozoa taken from males with a history of unexplained infertility.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia sebagai sebahagian daripada keperluan untuk Ijazah Master Sains.

IN VITRO FERTILIZING OF HAMSTER OVA AS AN AID IN ASSESSING THE FERTILIZING CAPACITY OF HUMAN SPERMATOZOA

oleh

Syed Zulkifli bin Syed Mohamed

Oktober, 1985

Penyelia : Prof. Madya Dr. Tuan Ariffeen Bongso

Fakulti : Kedoktoran Veterinar dan Sains Peternakan

Kajian telah dijalankan dibeberapa makmal penyelidikan untuk memeriksa keupayaan persenyawaan sperma orang lelaki mandul yang tidak diketahui sebabnya (unexplained infertility). Oleh kerana ova manusia sukar diperolehi, ova hamster telah digunakan sebagai ganti dalam kajian-kajian ini. Penyelidikan sedang dijalankan untuk mengesahkan assei penembusan sperma (sperm penetration assay) sebagai satu kaedah diagnostik yang boleh dipercayai bagi mengetahui interaksi sperma-ova dalam manusia dan haiwan.

Satu kajian telah dijalankan untuk mewujudkan suatu sistem assei untuk menilai punca kemandulan yang tidak dapat diketahui sebabnya pada lelaki di Lembaga Penduduk dan Pembangunan Keluarga

Negara, Kuala Lumpur. Ciri-ciri air mani dari 175 pancutan daripada 35 orang penderma (1 pancutan/penderma/minggu) menunjukkan 10 orang penderma subur dengan parameter-parameter mani yang normal sementara 25 penderma mandul yang mempunyai azoospermia (4%), oligospermia (44%), kiraan sperma rendah (32%), normozoospermia (12%) dan polyzoospermia (8%). Sepuloh daripada penderma mandul yang mempunyai parameter mani dalam lingkongan normal telah digunakan untuk assei penembusan sperma. Dalam assei ini tiga puloh hamster betina dewasa (Masocricetus auratus) telah disuntik, melalui intrapenitoneum, dengan 30 IU gonadotropin serum kuda bunting (pregnant mare's serum gonadotrophin) pada hari selepas estrus disusuri 48 jam kemudian dengan 30 IU gonadotropin chorion manusia (human chorionic gonadotrophin (HCG) untuk menghasilkan superovulasi. Basuhan dari uterus dan salur ovum, 15-17 jam selepas suntikan HCG, dapat menghasilkan 30.0±1.03 ova (20-43) dari tiap-tiap seekor hamster. Medium Ham F-10 (HF-10) didapati sesuai untuk menampong ova dan persenyawaan in vitro. Untuk mendapatkan ova tanpa zona, sel kumulus dan lapisan zona-pellusida telah disingkir menggunakan 0.1% hyaluronidase untuk 5-6 minit pada suhu 37°C dan 0.1% trypsin untuk 2-3 minit pada suhu 37°C. Lapan puloh peratus ova tanpa zona telah disenyawakan dengan sperma (1.5×10^6 sperma/ml) dari penderma subur, setelah mani itu dicuci dengan medium HF-10 yang telah diinkubat pada suhu 37°C selama 6 jam ($P<0.05$). Kadar penembusan adalah lebih rendah ($P<0.01$) pada ova yang masih dikelilingi oleh zona dan sel kumulus dengan masa prainkubasi sperma antara 3 hingga 9 jam sebelumnya. Apabila masa prainkubasi

sperma ditetapkan selama 6 jam dan ova tanpa zona dibiarkan bersatu dengan sperma selama 6 jam kadar penembusan maksima (80.0±2.9%; $P<0.05$) telah diperolehi. Kadar penembusan pada ova-ova yang masih mempunyai sel-sel kumulus adalah lebih rendah ($P<0.01$), jika dibandingkan dengan ova tanpa zona, jika interaksi sperma-ova dibiarkan berlaku selama 6 jam. Penembusan ova tanpa zona tidak terdapat pada 60% daripada penderma mandul yang mempunyai ciri-ciri mani yang normal, walaupun pancutan tiap-tiap minggu dari penderma digunakan selama 5 minggu berturut-turut. Analisa kromosom boleh dilakukan keatas 95% ova yang telah ditembusi sperma dan kehadiran set-set kromosom haploid sperma manusia didalam ooplasma hamster dijadikan sebagai pengesahan penembusan. Kajian ini menunjukkan bahawa zona pellusida ova hamster bertindak sebagai penghalang kepada penembusan oleh sperma manusia dan ova hamster boleh digunakan sebagai pengganti ova manusia untuk menguji keupayaan persenyawaan sperma lelaki yang mempunyai histori kemandulan yang tidak dapat diketahui sebabnya.

INTRODUCTION

Surveys of patients attending the fertility clinics of the National Population and Family Development Board, Kuala Lumpur showed that a large percentage of couples unable to have children were diagnosed as clinically normal. In the routine assessment for infertility in the male partners of these couples the fertilizing potential of their spermatozoa is evaluated for standard sperm parameters such as sperm counts, motility and morphology. Of these couples, the males had normal sperm counts, sperm morphology and sperm motility and over 70% of patients were diagnosed as "unexplained infertility" (Hamid Arshat, 1983). However, such semen evaluation for infertility has been considered imprecise (Sherins et al, 1977) and the validity of its use has been frequently questioned by clinicans, when sperm number and motility are within normal limits.

The fertilizing capacity of mammalian spermatozoa could be assessed more accurately by more recent methods. One method is by depositing the spermatozoa in the female genital tract at the time of ovulation and later examining the ova for fertilization. This method involves complicating factors inherent with *in vivo* techniques. Alternatively, more precise analytical studies of the fertilization process and sperm physiology could be carried out by insemination of ova *in vitro* (Edwards et al, 1969; Overstreet and Hembree, 1975; Seitz et al, 1971; Soupart and

Strong, 1974). For this technique, human ova and ova from proven domestic animals are not readily available in most hospitals and institutions. However, Barros et al, (1979) demonstrated that at the ultrastructural level, the human sperm nucleus after entry into the ova of hamsters, decondenses and transforms into a male pronucleus, typically identical with that of normal fertilization. Further, Rogers et al (1979), reported that the human spermatozoon fuses with the hamster egg vitelline membrane and decondenses with efficiencies related to presumed in vitro fertilization of males. This was further supported by the fact that the major barrier to interspecies fertilization in mammals appeared to be the acellular zona-pellucida, a glycoprotein-rich layer surrounding the vitellus at ovulation. Furthermore, sperm from infertile patients did not penetrate zona-free hamster eggs with the same frequency as did samples from normal donors. Based on this evidence the "hamster ova penetration assay" was developed in some laboratories and considered an important and reliable diagnostic test for examining capacitation and the ability of human spermatozoa to penetrate mammalian ova (Yanagimachi et al, 1976). Several workers have adopted the sperm penetration assay (SPA) for the diagnosis of male infertility (Barros et al, 1978; 1979; Hall, 1981; Karp et al, 1981; Overstreet et al, 1980; Rogers et al, 1979). It is possible that a large percentage of male patients with "unexplained infertility" attending the clinics of the National Population and Family Planning Board in Kuala Lumpur, carry spermatozoa that are unable to penetrate or fertilize mammalian ova. Further, such studies have not been carried out in Asian countries and it is

not known whether the spermatozoa of Asian behave differently to hamster ova. The results of such an assay would also shed light to the causes of such a high percentage of males with unexplained infertility.

This study was therefore aimed at developing the hamster ova test system and evaluating a group of patients for infertility using the developed test system. The main objectives of the study were: (1) to test the reliability of a superovulation procedure (Johnson and Alexander, 1984) in hamsters so as to obtain as many ova per animal; (2) to evaluate the semen of a group of human male patients with "unexplained infertility" using the conventional semen evaluation tests so as to identify 'infertile' males with normal semen characteristics; (3) examine the sperm penetration capabilities of such 'infertile' patients on hamster ova; (4) unravel and characterise the human haploid sperm chromosome constituents to confirm penetration and explore the possibility of studying genetic defects carried by human spermatozoa.

LITERATURE REVIEW

Human infertility

The subject of human infertility and the contribution made to it by many factors is complex. Chandley (1979) classified the term infertility as to include both subfertility and absolute sterility. Biologically, infertility was implied as the diminished capacity for producing offspring and statistically, infertility has been observed as a reduction in the actual number of offspring produced. In the human, infertility can be classified clinically as (a) primary infertility - that is, the inability to have any children at all or (b) secondary infertility - that is the inability to have additional children after several years of trying. The major causes of female infertility which accounts for 50 to 70% of all infertility have been grouped as (1) infections and resulting damage or blockage of the oviducts (2) hormonal or ovulation disorders and (3) endometriosis (growth of endometrial tissue outside the uterus). Much less is known about male infertility partly because men are less likely to seek full examination or treatment. Male infertility results primarily from low sperm concentrations or sperm abnormalities (Sherris and Fox, 1983).

In men, untreated genital infection causes infertility by creating inflammation or blockage of the reproductive tract. Such infection is usually caused by the sexually transmitted diseases

such as gonorrhea, chlamydial infection and mycoplasmas. These infections cause an urethritis which if not treated spreads to the vas deferens and eventually the epididymis. An epididymitis may result in scarring that partially or completely blocks sperm transport (Berger et al, 1979; Nilsson et al, 1968). Other diseases that cause male infertility are tuberculosis, filariasis, leprosy, mumps and schistosomiasis (Sherris and Fox, 1983).

In addition to disease, a variety of factors may impair sperm production and so cause male infertility. These include hormonal and genetic anomalies and exposure to external influences that impair sperm production. Best understood are the effects of severe hormonal abnormalities caused by genetic disorders. Low testosterone levels do not promote sperm production thus resulting in low sperm counts. Genic and chromosomal factors could be grouped and infertility then defined according to specific criteria. Penrose (1963) suggested three major groupings. In the first group, he placed those individuals who carry a'lethal condition' and who are infertile because they do not survive to reproductive age. They are killed by the genes or chromosomal abberations that they carry which are viewed as causes of pregnancy wastage exemplified by miscarriage, stillbirth, neonatal and infant deaths. The chromosomally abnormal, lethal and sublethal types in this group include most autosomal trisomies, triploidy and aneuploidy.

In the second group were placed those individuals who are infertile due to hereditary, mental or physical disorders that exclude them from establishing a normal heterosexual relationship.

Such individuals include the majority of Down's syndrome patients and other autosomal and sex chromosomal aneuploids that lead to the necessity of confining the individual in an institution. In the third group, Penrose (1963) placed those individuals whose general health was not seriously disturbed by the abnormalities they carry but show infertility through genic or chromosomal conditions which affect the gonads. These individuals are likely to marry but their childlessness condition may lead them to seek advice at infertility clinics.

In one of the earliest surveys on human infertility in 1957, Ferguson-Smith et al, examined the sex chromatin of 91 high-grade subfertile males attending a Glasgow Clinic and found 10 to be chromatin positive and clinically classified as Klinefelter's syndrome. They concluded that Klinefelter's syndrome accounted for 11% of all cases of high grade subfertility in males. Investigations on infertility problems indicated that the male partner was responsible for 30-50% of the problem couples (Opitz et al, 1979). Chandley et al (1975) reported that the frequency of chromosomal abberations was 6.2% among males with a sperm count less than 20 mill/ml and it rose to 15.3% among azoospermic men. Van Niekerk (1978) also reported an increasing freqnency of chromosomal changes with declining sperm count. He observed a 11.5% incidence among azoospermic men versus 9.1 percent among oligospermic males (< 10 mill/ml). The available literature on chromosome abnormalities leading to male infertility are summarised in Table I and the most common anomaly was the 47XXY Klinefelter's syndrome (Chandley et al, 1975); 46 XX males (Fraccaro et al, 1979)

TABLE I

CHROMOSOMAL ANOMALIES AND INFERTILITY

Investigation	No. of patients	Sex chromosome abnormalities	Autosome abnormalities	Variants	% Chromosomal abnormalities (exact. variants)
Van Wijck et al, 1963	29	4	-	-	13.8
McIlree et al, 1966	50	2	2	-	8.0
Philip et al, 1970	98	7	-	1	7.1
Dutrillaux et al, 1971	40	4	2	-	15.0
Luciani et al, 1972	186	20	6	5	14.0
Millet et al, 1972	100	5	-	3	5.0
Stenchever and Jarvis, 1971	31	1	-	-	3.2
Kjessler, 1972	1263	-	84*	-	6.6
Koulischer and Schoysman, 1974	200	20	2	4	13.0
Hendry et al, 1976	198	4	3	21	3.5
Chandley et al, 1975	1599	22	13	27	2.2
Rao and Rao, 1977	117	9	-	-	7.7
Thomas and Thomas, 1978	32	6	-	-	18.7
Van Niekerk, 1978	234	17	6	-	9.8
Faed et al, 1979	348	3	10	-	3.7
Peter et al, 1980	102	9	-	5	8.8
Joseph and Thomas, 1982	43	10	1	2	25.6

*Overall chromosomal abnormalities